INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: METHOD AND KIT FOR CANCER DIAGNOSIS

(57) Abstract

An assay method for the diagnosis of cancer in an individual is provided in which human sera samples are contacted with assay compositions, each comprising a single mutant p53 protein or a plurality of different mutant p53 proteins. An immune reaction, which then develops in the sera with the mutant protein, is assayed which provides a basis for diagnosing whether the tested individual has cancer.

METHOD AND KIT FOR CANCER DIAGNOSIS

THE FIELD OF THE INVENTION

The present invention is generally in the field of cancer diagnosis and provides a method and a kit for the diagnosis of cancer based on the detection of anti-p53 antibodies in suspected individuals.

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PRIOR ART

The following references are related to the present invention.

1.   Lane, D.P., and Crawford. L. (1979). Nature 278, 261.263.

10   2.   Linzer, D.I.H., and Levine, A.J. (1979). Cell 17, 43-52.

3.   Rotter, V., Witte, O.N. Coffman, R., Baltimore, D. (1980). J. Virol. 36, 547-555.

4.   Rotter, V., Boss, M.A. and Baltimore, D., (1981). J. Virol. 38, 336-346.

5.   Rotter. V. (1983). Proc. Natl. Acad. Sci. 80, 2613-2617.

6.   Eliyahu. D.. Raz. A., Gruss. P., Givol, D.. Oren M. (1984). Nature 312, 646-649.

7.   Jenkins, J.R., Rudge. K. and Currie. G.A. (1984). Nature 312. 651-654.

8.   Parada, L.F., Land, H., Weinberg. R.A., Wolf, D. and Rotter, V. (1984). Nature 312. 649-651.

9.   Wolf D, Harris N, Rotter V. (1984). Cell 38, 119-129.

10. Eliyahu. D., Michalovitz, D., Eliyahu. S., Pinhasi-Kimhi. O. and Oren. M. (1989). Proc. Natl. Acad. Sci USA 86, 8763-8767.

11. Finlay, C.A.. Hinds. P.W. and Levine. A.J. (1989). Cell 57, 1083-1093.

12. Kelman, Z., Prokocimer. M., Peller, S., Kahn, Y., Rechavi. G., Manor, Y.. Cohen A.. and Rotter, V. (1989). Blood 74, 2318-2324.

13. Nigro, J., M., Baker, J.S., Preisinger. A.C, Jessup. J.M., Hostetter, K., Cleary, K., Bigner, S.H. Davidson, N., Baylin. S., Devilee P., Glover. T., Collins, F.S., Weston. A., Modali, R., Harris, C.C., and Vogelstein, B. (1989). Nature 342, 705-708.

14. Baker. J.S., Fearon. E.R.. Nigro, J.M.. Hamilton, S.R., Preisinger. A.C., Jessup. J.M., van Tuinen. P.. Ledbetter, D.H.. Barker, D.F., Nakamura. Y.. White. R.. and Vogelstein. B. (1989). Science 244, 217-221.

15. Takahashi, T., Nau M.M., Chiba. I. Birrer. M., J. Rosenberg R.K., Vinocour. M.. Levitt. M.. Pass. H., Gazdar, A.F., and Mina J.D. (1989). Science 246, 491-494.

16. Mercer. W.E., Shields. M.T., Amin. M.. Sauve, G.J., Appella, E., Romano, J.W. and Ullrich, S.J. (1990). Proc. Natl. Acad. Sci. USA 87, 6166-6170.

17. Vogelstein, B. (1990). Nature 348, 681-682.

18. Levine, A.J., Momand, J., and Finlay, C.A. (1991). Nature 351, 453–456.

19. Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V. and Vogelstein, B. (1990). Science 249, 912–915.

5   20. Rodrigues, N.R., Rowan, A., Smith, M.E.F., Kerr, I.B., Bodmer, W.F., Gannon, J.V. and Lane, D.P. (1990). Proc. Natl. Acad. Sci. USA, 87, 7555–7559.

21. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C., (1991). Science 253, 49–53.

10   22. Wolf, D., and Rotter, V., (1985). Proc. Natl. Acad. Sci. 82, 790–794.

23. Prokocimer, M., Shaklai, M., Ben-Bassat, H., Wolf, D., Goldfinger, N., Rotter, V., (1986). Blood 68, 113–118.

24. Ahuja, H., Bar-Eli, M., Advani, S.H., Benchimol, S., and Cline, M.J. (1989). Proc. Natl. Acad. Sci. USA 86, 6783–6787.

15   25. Crawford, V.L., Pin D.C., and Bulbrook, R.D. (1982). Int. J. Cancer 30, 403–408.

26. Studier, F.W. and Moffet, B.A. (1986) J. Mol. Biol. 189, 113–130.

27. Rosenberg, A.H., Lade, B.N., Chui, D.-S., Lin, S.-W., Dunn, J.J. and Studier, F.W. (1987) Gene 56, 125–135.

20   28. Mackett, M., G.L. Smith and B. Moss (1985). In: DNA Cloning, Volume II (D.M. Glover, ed.), IRL Press, Oxford/Washington, D.C., pp. 191–211.

29. Graham, F.L. and Van der Eb, A.J. (1973). Virology 52, 456–467.

30. Shohat-Foord O., Bhattacharya, P., and Rotter V., (1991), Acid Research 19, 5191–5198.

31. Yewdell, J.W., Gannon, J.V. and Lane, D.P. (1986). J. Virol. 59, 444–452.

32. Gannon J.V., Greaves, R., Iggo, R., and Lane, D.P. (1991). EMBO J. 9, 1595–1602.

33.   Ronen. D., Teitz. Y.. Goldfinger, N.. and Rotter, V. (1992). Nucleic
Acids Research, Vol. 20, 13, 3435-3441.

The above references will be referred to herein by indicating
their number in the above list within brackets.

BACKGROUND OF THE INVENTION

p53 is a nuclear protein having a molecular weight of about 53
kilodalton and is a cell cycle suppressor.   When p53 was first discovered,
more than a decade ago, it was described as a cellular phosphoprotein that
is overproduced in tumor cells (1-4) and forms a complex with the viral
large T antigen. (1-2) Analysis of a variety of cell lines and primary
tumors, have indicated that p53 is overproduced in a large number of
primary tumors and established cell lines and thus it was suggested that p53
could be defined as a tumor-specific marker. (5)

Association of the p53 protein with the malignant process was
initially deduced from observations that the protein is stabilized in tumor
cells and that it occasionally forms complexes with tumor specific antigens.
(1-2) This was further substantiated when it was found that the p53 gene
isolated from tumor cells. codes for a protein that facilitates the malignant
process. (6-9) As investigation of the p53 proceeded, it became clear that
mutant forms p53 protein are overproduced in tumor cells, while in normal
cells the wild type p53 is expressed at low molar concentrations. (10-16)

Inactivation of the p53 gene through deletion or mutation.
plays a critical role in the development of malignant transformation probably
allowing a cell to escape normal growth control. (17-18) In human
colorectal carcinoma the transition from the benign to the malignant state
correlates with the loss of the two wild type alleles for p53, indicating a
tumor suppressor function for p53. (13-14) Allelic degeneration of p53 was

also found in human carcinoma of the lung (15) and in a number of
commonly occurring types of carcinoma. (19-21) In most of the carcino-
mas, mutant p53 protein forms are overexproduced whereas in other
malignancies, mostly those derived of myeloid origin, the p53 gene is
5   rearranged and no p53 protein is found. (22,23,12,24)

There has been a single report (25) showing that 14% of breast
cancer patients exhibit variable titers of anti-p53 antibodies. This rather low
frequency of anti-p53 antibodies was unpromising for the purpose of the
development of immunoassay for the diagnosis of cancer.

10   It is the object of the present invention to provide an
immunoassay method for the detection of cancer in suspected individuals.

It is a further object of the invention to provide a kit for the
detection of cancer in suspected individuals.

15   GENERAL DESCRIPTION OF THE INVENTION

The present invention is based on the realization that mutant
p53 proteins (hereinafter "m-p53") which are accumulated in the tumor cells
are immunogenic and induce an immune reaction resulting in the production
of specific anti-p53 antibodies that can be detected in the serum by means
20   of interaction with mutant p53 proteins.

Thus, in accordance with the present invention there is
provided an assay method for the diagnosis of cancer in an individual based
on the detection of anti-p53 antibodies, comprising:

(a)   contacting human sera samples with assay compositions
25   comprising each a single mutant p53 (m-p53) protein or a plurality of
different mutant p53 proteins; and

(b)   determining the degree of anti-m-p53 immune reaction in the
individual by either detecting the level of binding of antibodies in an
appropriate test sample to said m-p53, detecting the level of cellular

immune reaction in another appropriate test sample, or by a combination of the two detections, an immune reaction above a certain level indicating that the individual is suspected of having cancer.

Thus, by one embodiment of the invention the immune reaction which is being determined is an antibody-antigen reaction ("antibody embodiment") while by another embodiment the immune reaction which is being determined is a cellular immune reaction ("cellular embodiment"). The antibody embodiment is currently preferred in accordance with the present invention as it is generally easier to carry out than the cellular embodiment and involves techniques which are more familiar and more readily available to technicians in clinical laboratories. Both the antibody embodiment and the cellular embodiment can be performed by a large number of standard procedures readily familiar to the artisan.

The invention will hereinafter be described with reference to the antibody embodiment, it being understood that it is not limited thereto, and that the description applies, mutatis mutandis, also to the cellular embodiment.

In the performance of the assay by the antibody embodiment of the invention, said test sample is contacted with said assay composition and the binding of the antibodies in the test sample to the proteins in the assay composition is measured. The m-p53 in the assay composition may be suspended in an aqueous medium or bound to a solid support such as beads, microbeads, gel particles, the wall of microwells, etc. Measurement of binding of antibodies in the test sample to the m-p53 in the assay composition may be carried out by any number of means known per se such as immunoprecipitation, radio-immunoassay (RIA), fluorescence-immunoassay (FIA) enzyme-linked immuno-sorbent assay (ELISA), etc. The chosen means for the measurement will determine the exact nature of the assay composition (whether the m-p53 are in suspension or bound to a solid

support, and in the latter case or the nature of the solid support), and vice versa.

The assy composition may comprise a single m-p53 type or a plurality of different types of m-p53. Where the assay composition comprises a single type of m-p53, typically a plurality of test samples from the same individual will be used, each contacted with a different assay composition, each of which comprises a different type of m-p53. Where the assay composition comprises a plurality of different types of m-p53, a single assay composition may at times be sufficient for carrying out the test. Although, when using assay composition comprising a plurality of m-p53 the identity of the protein to which the antibodies bind cannot be determined, such knowledge is generally unimportant for the purpose of cancer diagnosis.

As will be appreciated by the artisan, it is preferable to perform the test with several repetitions of the same assay composition - test sample couples, or to minimize false positive and false negative results which may occur as a consequence of the natural biological variability inherent in such reactions. Furthermore, the assay is preferably performed with several dilutions of the assay composition which is necessary since the antibody titer in the sample is as a rule, not known beforehand.

The present invention also provides assay compositions comprising a single m-p53 protein or a mixture of different m-p53 proteins, for use in the above method. The assay composition may be provided as an aqueous suspension, as a dry composition, e.g. a lyophilizate, which is supplemented with an aqueous medium prior to performing the assay or may be provided as bound to a solid support, such as walls of microwells, beads, microbeads, gel particles etc.

The m-p53 to be used in accordance with the invention may be any mutant p53 protein, such as those isolated from bacterial, e.g. *E.Coli*,

cells, as described in Shohat-Foord et al. (30). Other mutant p53 proteins can be expressed by Vaccinia viral vectors as described hereinbelow and in Ronen et al. (33). More common and less common m-p53's can be used. Generally, by increasing the number of the different types of m-p53 proteins used in the method, the accuracy of cancer diagnosis in accordance with the invention will increase.

In order to obtain various m-p53, cDNA encoding m-p53 may be isolated from different tumors and then cloned and expressed by various known cloning methods. Alternatively, wild type or m-p53 gene may be isolated and site directed mutated in random by various means and then cloned and expressed. The m-p53's are isolated and then used in accordance with the invention. It is appreciated that by the last approach, the m-p53 mixture may include also a large number of such proteins which may not occur naturally, but this by itself should have no adverse affect on the assay.

The present invention further provides a serological kit for the diagnosis of cancer in accordance with the above method comprising:

(a)   a plurality of assay compositions each comprising a different type of m-p53 or one or more assay compositions comprising a plurality of different types of m-p53;

(b)   reagents which are required for detecting and/or measuring the level of binding of antibodies to proteins in said composition; and optionally,

(c)   instructions for use in said method.

An example of the kit is one comprising microtiter plates having a plurality of wells, the wells of which are coated with m-p53. The wells may be coated by a mixture of m-p53 or each one as a group of wells may be coated by a different m-p53.

In accordance with preferred embodiments, the serological anti-p53 defecting kit comprises a substantially purified and preferably defined mixture of m-p53 proteins expressed in *E.Coli* bacterial vector or vaccinia viral vectors. The complexes generated as a result of reaction of
5 the assay composition with a positive serum can be visualized by specific anti-human immunoglobulin reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the expression of p53 protein by recombinant vaccinia
10 viruses. HeLa cells were infected for 60 min., at M.O.I. 5 PFU per cell, with either wild type vaccinia virus (WR), vaccinia p53WT (p53CD) which is a recombinant vaccinia vector coding for wild type p53, or vaccinia p53mut (p53M8) which is a recombinant vaccinia vector coding for a mutant p53 protein. 20 hrs after infection the cells were metabolically labelled for
15 1 hr with ^{35}S-methionine and protein content was analyzed. Total cell lysates, 5×10^5 cpm prior to immuno-precipitation (A) were compared to specific immuno-precipitated product binding the PAb-242 monoclonal antibodies (B).

Fig. 2 shows the results of a Western blot analysis. HeLa cells were
20 infected with either vaccinia p53WT or vaccinia p53mut and at indicated time points, cells were harvested and reacted with the anti-p53 monoclonal antibody PAb-242. UI, cell lysates obtained from uninfected HeLa cells; BV, cell lysates obtained from HeLa cells 24 hrs post-infection with WT vaccinia virus; M, markers indicating the positions of molecular weight
25 standards.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described with reference to several specific embodiments it being understood that the invention is not limited thereto.

5

EXAMPLES

1. **Establishment of a p53 cDNA plasmid collection:**

    (a)   Isolation of m-p53 cDNA from fresh human tumors:

10   cDNAs clones coding for different mutant p53 proteins were isolated, subcloned into the pET-8c plasmid and expressed in *E.Coli.* as described in Shohat-Foord *et al.*, mentioned above. To increase the repertoire, additional types of p53 cDNA may be isolated from human primary tumors. To facilitate the isolation of different types of p53 cDNA

15   from different human primary tumors, the PCR system to amplify the entire open reading frame of 1256 bp can be used. Such PCR amplified cDNAs obtained are directly cloned into the various expression vectors.

For the cDNA preparation total mRNA is extracted from different fresh tumor biopsies. An alliquot of 5μg of RNA is reverse

20   transcribed in a 20μl reaction vessel using reverse transcriptase (Molecular Genetics Resources. Tampa Florida). 5μl of cDNA are added to 100μl primer extension reaction mixture which contains 0.25mM of each primer (the primers are oligonucleotides 17-mer in size synthesized according to known p53 sequence), 200 mM of each deoxynucleotide triphosphate with

25   2 units of Vent DNA polymerase with appropriate buffers (N.E. Biolabs, Bevery, MA). 5-35 PCR cycles are carried out. 10μl of the reaction mixture are then run on a 1% agarose gel to visualize amplified DNA and to estimate yield. 10μl of the amplified reaction mixture are incubated at 37°C for 30 min with 1 unit of T4 DNA polymerase (Boehringer Mannheim.

GmbH, Germany) in order to create blunt ends in the amplified DNA. The primer may also be designed to code for specific cloning sites such as BamHI direct cloning sites. Amplified DNA is electrophoresed on a 1% agarose gel, the appropriate band is cut out and DNA is extracted and
5   purified using Geneclean™ Kit (Bio 101, La Jolla CA).

(b)   Site Directed mutagenesis of the p53 antigen:

As preliminary antigens, p53 proteins which are mutated in the central part of the p53 molecule may be used. The observation that most mutant human p53 proteins are recognized by the PAb-240 monoclonal
10   antibody (31,32) suggest that in spite of major changes in the protein structure most m-p53 share certain antigenic features. For site-directed mutagenesis, human p53 cDNA that have been isolated, e.g. from a λgt10 library of SV40 transformed cells, may be cloned as BamHI fragments into the pBluescript KSII+ vector (Stratagene). Single-stranded DNA isolated
15   from the library serve as a template for mutagenesis. Oligonucleotide-directed site-specific mutagenesis may be performed essentially according to the Bio-RAD MUTAGENE™ kit protocol, using single-stranded DNA, which may be produced in the E.Coli strain, CJ236, as a template. The specific oligonucleotides for mutagenesis may be synthesized and purified
20   by fractionation on a polyacrylamide gel. The extension reaction may be carried out using T4 DNA polymerase and T4 gene 32 product (Boehringer Mannheim, FRG), transfected into E.Coli TG1 competent bacteria. Mutated plasmids are analyzed by DNA sequencing using the Sequenase V.2 system (USB Cleaveland, OH).

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2.   Subcloning of mutant p53 cDNAs into expression vectors:

(a)   Bacterial expression vector:

Construction of a p53 expression plasmid can be performed, for example, by insertion of p53 cDNAs into the pET-8c plasmid (26-27)

in which case the expression of the inserted p53 cDNA is controlled by the T7 polymerase promoter. As another example, p53 cDNA may be inserted into a modified pET12 vector.

When utilizing the pET-8c vector, all cDNAs are cloned as
5   NcoI-BamHI fragments into the same site. The first ATG, the start codon of the native protein, lies within the NcoI cloning site.

Bacterial cells, e.g. XL-1 blue (Stratagene), are transformed with the plasmids. Such bacterial cells containing the different plasmids may be grown in 37°C LB medium containing ampicillin (50 mg/ml). IPTG
10 at a final concentration of 0.4mM is added when the cells reach about 1 O.D. Addition of IPTG to a growing culture of *E.Coli* B strain BL21(DE3) lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid. A certain time after the induction, e.g three hours, cells are harvested, e.g. by centrifugation, and the pellet is washed and
15 resuspended, e.g. in TE containing 50mM NaCl and 10mg/ml lysozyme. The lysate is sonicated, e.g. for 3 x 15 sec, and subjected to low speed centrifugation. The pellet contained the total membrane fraction, and the supernatant contained the soluble fraction. Insoluble material containing the p53 protein is washed, e.g. three times with 4M urea 0.1M Tris-HCl pH 8.5,
20 and is solubilized, e.g. by dissolving in 7M guanidine-HCl, 50mM Tris-HCl pH 9.0, 2mM EDTA. Finally, the cell lysates are clarified and dialyzed against 50mM NaCl, 10mM Tris-HCl pH 7.8 and 1mM EDTA.

In accordance with the above procedure, *E.Coli* strains expressing mutants of the p53 proteins are obtained as reported in Shohat-
25 Foord *et al.* (30), the context of which is incorporated herein by reference.

(b)   Vaccinia expression vector:

pSVL-CD coding for the wild type p53 protein and pSVL-M8 coding for the mutant p53 protein, were used as sources for construction of p53 recombinant vaccinia viruses. The two cDNA inserts were isolated, e.g.

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by digestion with BamHI. and subcloned. e.g into a BamHI site of vaccinia
virus vector, such as pgpt-ATA-18 carrying a xanthine guanine
phosphoribosyl transferase gene (gpt) for selection (the vector is available
from D. H. Stunnenberg of EMBL). Transfer of the p53 genes from the
5   recombinant plasmids to the wild type vaccinia virus WR was achieved by
using a modification of the standard homologous recombination meth-
od (28). Briefly, TK⁻143 cells are infected with wild type vaccinia virus WR
strain at a multiplicity of infection (M.O.I.) of 0.1 PFU per cell. At 2 h
post-infection. the · cells were transfected by the calcium phosphate
10   precipitation procedure (29), with 1 μg of pgpt-ATA-18 plasmid DNA either
carrying p53CD or p53M8 coding sequences and 2 days later, recombinant
viruses were collected from the infected cells and plaque-purified on RK₁₃
cells overlaid with 1% agarose in a growth selective medium (DMEM
supplemented with 10% FCS and 20 μg/ml mycophenolic acid. 150 μg/ml
15   xanthine and 15 μg/ml hypoxanthine); Individual recombinant plaques were
purified by three rounds of plaque purification and amplified on RK₁₃ cells.

Following the above procedure, recombinant viral stocks
carrying either the wild type p53 cDNA, designated vaccinia p53WT
(containing the p53CD coding for the wild type p53 protein) or the mutant
20   p53 cDNA designated vaccinia p53mut (containing the p53M8 coding for
mutant p53 protein) were prepared from the amplified cultures and titrated
for the amount of virus PFU before use. The · iral stocks were successfully
utilized in the transfection of wells to produce wild type and mutant p53
proteins.

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3.   **Preparation of infected cell lysate: immunoprecipitation and
immumoblotting**

Monolayer cultures were infected with vaccinia p53 WT,
vaccinia p53 mut or with parental vaccinia virus WR vaccinia vector at a

M.O.1. of 5 PFU per cell of each virus. 24 h post-infection, the cells were metabolically labelled with 0.125 mCi of ^{35}S-methionine (Amersham) for 1 h at 37°C in methionine-deficient (met⁻) Eagle's modified medium supplemented with 10% heat-inactivated dialyzed fetal calf serum. Cells

5   were lysed in lysis bufer: 50 mM Tris pH 7.5; 150 mM NaCl; 0.5% NP40; 0.5% deoxycholate; 0.01% SDS; 2mM PMSF, and pre-cleared with 10% fixed *Staphylococcus aureus*. Equal amounts of TCA-insoluble radioactive material were reacted with specific antibodies for 2 h at 4°C. The immune complexes were precipitated with 10% fixed *Staphylococcus*

10   *aureus* and washed 3X in PLB buffer: 10 mM NaH$_2$HPO$_4$ pH 7.5; 100 mM NaCl; 1% Triton X100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate. The immune complexes were separated on SDS-PAGE (Lammli, 1971). For immunoblotting, cells were lysed in sample buffer and subjected to PAGE, as above. The fractionated proteins were electrotransferred to

15   nitrocellulose membranes and the proteins were detected using the Protoblot western blot Ap system (Promega).

4.   Experimental results with vaccinia virus expression vector

In order to examine the p53 proteins expressed by the
20   recombinant viruses, HeLa cells, that lack almost any detectable endogenous p53 proteins, were chosen as a convenient cell system for measuring the expression of p53 protein introduced by the viral infection. To that end, HeLa cells were infected with either vaccinia p53WT, vaccinia p53M8 or the native vaccinia virus vector devoid of any foreign DNA. At 24 hours
25   post-infection, the cultures were labelled for 1 hr with ^{35}S-methionine, and equal amounts of trichloroacetic (TCA) - insoluble radioactivity of cell lysates were reacted with PAb-242 (31) anti-p53 monoclonal antibodies which recognize both wild type p53 and mutant p53 proteins. Fig. 1 represents cellular and viral specific proteins synthesized in the cells infected

with the various recombinant viruses. Lanes A in Fig. 1 show the pattern of total protein expressed in the cells. At that level of resolution it was clear that the cells infected by vaccinia p53WT or vaccinia p53mut, expressed an additional band of the expected p53 product. Upon immunoprecipitation

5   with a specific anti-p-53 antibodies, the expected corresponding p53 proteins were immunoprecipitated only from cultures infected with recombinant vaccinia virus, and no specific immunoprecipitation occurred from cultures infected with the vaccinia virus vector alone.

In order to detect the earliest time point for maximum

10 production of the recombinant p53 proteins expressed after infection, the rate of p53 synethesize at various time intervals after infection was measured. HeLa cells were infected with recombinant wild type p53 or mutant p53 vaccinia viruses. At times indicated in Fig. 2, the cells were harvested, cell lyses were analyzed by the western blot technique using PAb-242 specific

15 anti-p53 antibodies. As can be seen in Fig. 2, the synthesis of recombinant wild type p53, as well as that of recombinant mutant p53 proteins, is detectable as early as 8 hrs after infection. 30 hours following infection, at the level of p53 proteins platode and remained as after 24 hrs (data not shown).

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5.   Large scale purification of m-p53 antigen from bacteria:

Secreted m-p53 produced in vectors, such as the pET 12 vector commercially available (Novagene) is purified as a correctly folded protein, using either immunoaffinity or an alternative route, or a combination

25 of the two.

Non-secreted m-p53 produced in vectors, such as the pET3 vector commercially available (Novagene) is purified as inclusion bodies. These are dissolved using 6.5M urea and the denatured m-p53 protein is correctly refolded by dialysing the solution against decreasing concentrations

of urea and NaCl and increasing DTT concentration. Incorrectly folded
molecules are removed by ultracentrifugation. The correctly folded protein
may be further purified using either immunoaffinity or a combination of
immunoaffinity with other chromatographic procedures:

(a) Immunoaffinity purification of the antigen:

Immunoaffinity purification may be carried out by utilizing
three anti-p53 monoclonal antibodies. Ascites fluids from female C₇Bl/6
mice injected interperitonally with hybridoma lines may be used as a source
for the antibodies. Antibody levels in these ascites fluids can reach 10-20
mg/ml. The following monoclonal antibodies (MAb) may be used:
monoclonal anti-p53 PAb240, PAb-242, PAb-246 (36,37); PAb-421 (38);
200.47 (39), and RA3—2C2 (40). MAb can be initially purified by affinity
chromatography on Protein A-Sepharose columns. Purified MAbs can be
bound covalently to an activated matrix (Affi-prep, BioRad), or to Protein
A-Sepharose. p53 proteins from difference sources can be affinity purified
by the use of one or more antibodies, as needed. Conditions for elution of
the p53 protein from each antibody are in accordance with standard
procedures.

(b) Alternative routes for purification of the m-p53 antigen:

Such routes can be employed if for some reason immunoaffinity-
purification of the antigen is not adequate, or as a preliminary purification
step, prior to the immunoaffinity-purification step.

For example, conditions for the purification of the murine p53
produced in *E.Coli* comprise the use of an ion exchange column (DEAE-
Sephacell; loading in 50 mM NaCl, elution in 250 mM NaCl) and an
affinity medium (phosphocellulose: loading in 20 mM KPO₄ pH 7.8 elution

in 300 mM of the same buffer). By modification of these conditions, human wild type and mutated p53 proteins may be purified.
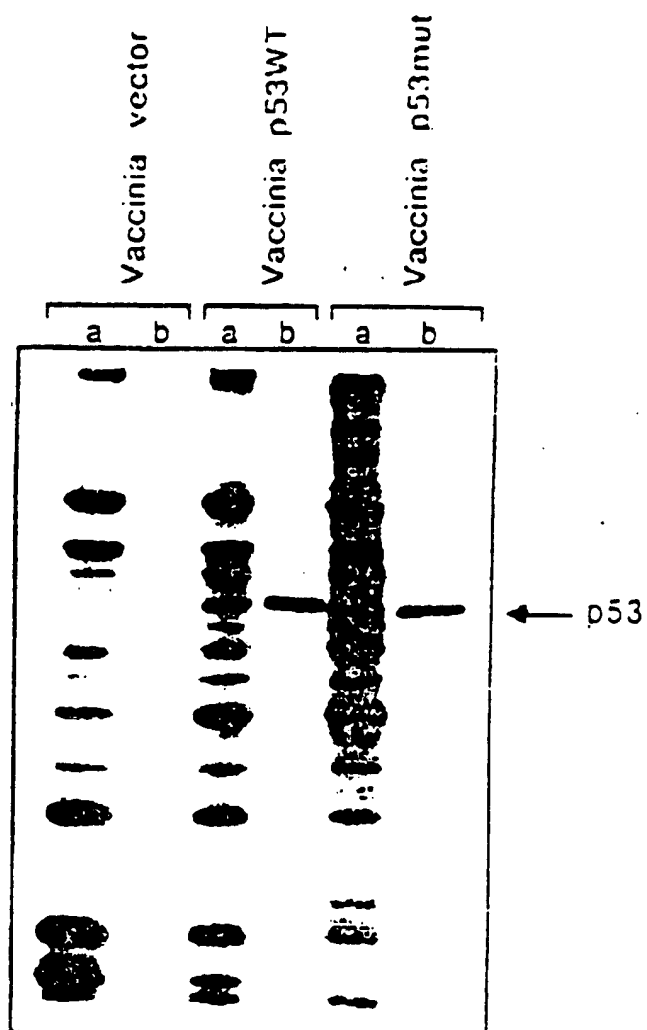

6. **Kits and assay method:**

5   The kit may consist of a microtiter plate whose wells are coated with a "broad p53 antigen", i.e. a plurality of different m-p53 proteins. Alternatively, each well or a group of wells may be coated with a different type of m-p53.

Serum (10-20ul) at serial dilutions, are applied to each well.

10   After a predetermined incubation period the plate is washed and goat anti-human peroxidase conjugated antibodies are applied to each well. As controls, the kit may contain serum from p53-antibody positive and negative patients and purified anti-p53 Ab for quantitative purposes.

CLAIMS:

1.   An assay method for the diagnosis of cancer in an individual,
comprising:

5   (a)   contacting human sera samples with assay compositions
comprising each a single mutant p53 protein or a plurality of different
mutant p53 proteins; and

         (b)   determining the degree of anti-mutant p53 immune reaction
in the individual by either detecting the level of binding of antibodies in an
10   appropriate test sample to said mutant p53, detecting the level of cellular
immune reaction in another appropriate test sample, or by a combination of
the two detections, an immune reaction above a certain level indicating that
the individual is suspected of having cancer.

2.   A method according to Claim 1, comprising determining the
15   level of anti-mutant p53 protein antibodies in the test sample.

3.   A method according to Claim 1, wherein the mutant p53
proteins are suspended in an aqueous medium.

4.   A method according to Claim 1, wherein the mutant p53
proteins are bound to a solid support.

20   5.   A method according to Claim 1, wherein said assay composi-
tions comprise each a single type of mutant p53 protein and the method
comprises contacting a plurality of test samples each with an assay
composition comprising a different type of mutant p53, and measuring the
immune reaction in said test samples separately for each assay composition.

25   6.   A method according to Claim 1, wherein said assay composi-
tion comprises a plurality of different types of mutant p53 proteins, and the
method comprises contacting one or more test samples with one or more of
said assay compositions, and measuring the immune reaction in said test
samples.

7.　　　A method according to Claim 6, wherein the mutant p53 proteins in the assay compositions are obtained from E.Coli cells transfected with a mutant p53 expression vector.

8.　　　A method according to Claim 6 wherein the mutant p53 proteins are obtained from cells transfected with a recombinant vaccinia virus vector, capable of expressing mutant p53.

9.　　　An assay composition for use in a method according to Claim 1.

10.　　　An assay composition according to Claim 9, comprising a plurality of different types of mutant p53 proteins.

11.　　　A serological kit for the diagnosis of cancer by the method of Claim 1, comprising:

　　　(a)　　a plurality of assay compositions each comprising a different type of mutant p53 protein or one or more assay compositions comprising a plurality of different types of p53 proteins; and

　　　(b)　　reagents which are required for detecting and/or measuring the level of binding of antibodies to proteins in said composition.

12.　　　A kit according to Claim 11, wherein said proteins are bound to a solid support.
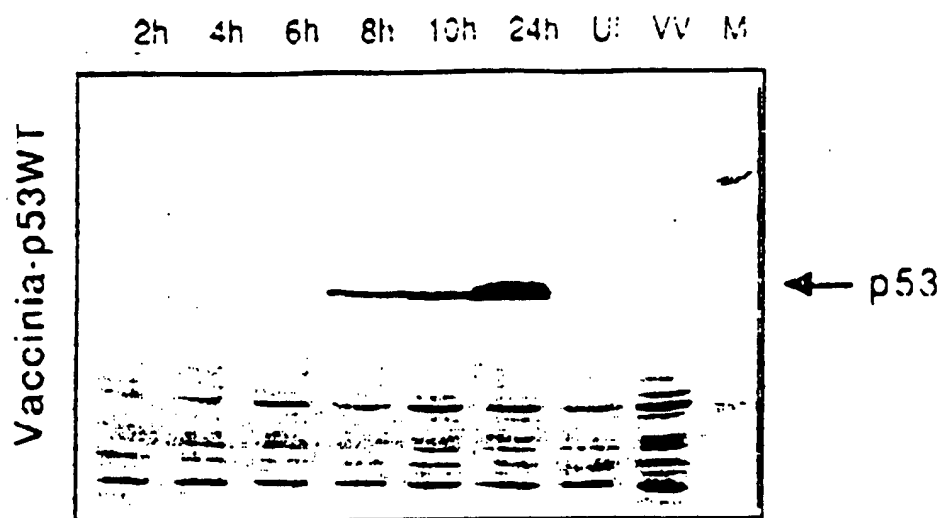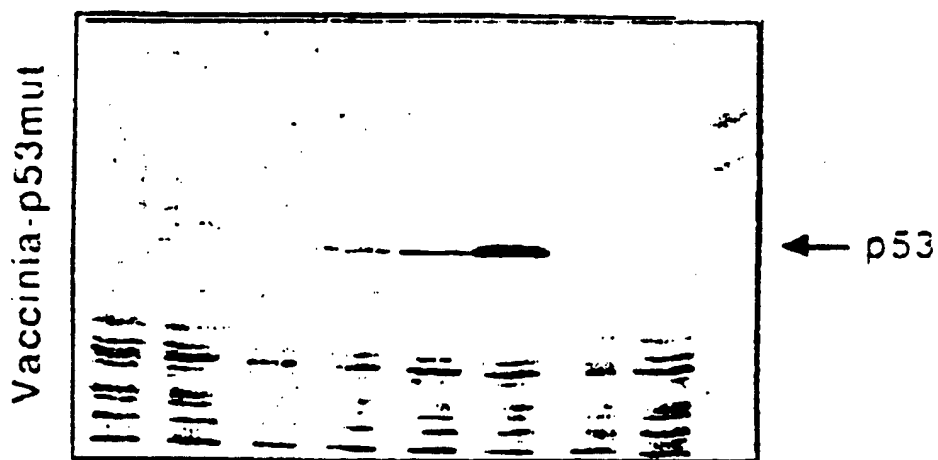
FIG. 1

FIG. 2a

FIG. 2b

International application No

PCT/US93/10411

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/574, 33/543
US CL :435/7.23, 975, 5; 436/64, 813, 518
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 975, 5; 436/64, 813, 518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: Biosis, CAB Abstracts, Embase, Medline, Cancerlit, Derwent
search terms: p53, antibod?, mutant?, mutation?, cancer, carcinoma, serum, sera

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Cancer Research, Volume 52, issued 01 August 1992, S.T. Winter et al, "Development of Antibodies against p53 in Lung Cancer Patients Appears to Be Dependent on the Type of p53 Mutation," pages 4168-4174, especially the bottom of the left-hand column of page 4170, the right-hand column of page 4170, and the middle of the right-hand column of page 4171.	1,2,4-6,9-12 ———— 3,7,8

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 January 1994	0 2 FEB 1994

Name and mailing address of the ISA/US	Authorized officer
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P — Y, P	Clinical Biochemistry (Canada), Volume 25, No. 6, issued December 1992, S. Hassapoglidou et al, "Antibodies to the p53 Tumor Suppressor Gene Product Quantified in Cancer Patient serum With a Time-Resolved Immunofluorometric Technique," pages 445-449 (ABSTRACT ONLY), see the entire abstract.	1,2,4,9 — 3,5-8,10-12
X — Y	Proc Annu Meet Am Assoc Cancer Res, Volume 33, issued 1992, G.E. Trivers et al, "Detection of Anti-p53 Antibodies in Lung Cancer Cases and Controls," Abstract No. A1745, see the entire abstract.	1,2,4,9 — 3,5-8,10-12
X, P — Y, P	British Journal of Cancer, Volume 65 (Supplement 16), issued December 1992, J.A. Green, "Serum Autoantibody to p53 in Breast Cancer Patients: Relationship to Tumor Differentiation," page 15, see the entire abstract.	1,2,4,9 — 3,5-8,10-12